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Nearctic walnut leafminers invade Europe: first *Coptodisca lucifluella* (Clemens, 1860) and now *Coptodisca juglandiella* (Chambers, 1874) (Lepidoptera, Heliozelidae)

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Abstract. The Nearctic leafminer of black walnut *Coptodisca juglandiella* (Chambers, 1874) was found in Hungary and reported as new for Europe. Larvae were found in leafmines on black walnut (*Juglans nigra* (L.)), white walnut (*J. cinerea* (L.)) and Arizona walnut (*J. major* (Torr.)); the latter two *Juglans* species are new host plant records for *C. juglandiella*. Mines of *Coptodisca lucifluella* (Clemens, 1860), another Nearctic invader, were found on leaves of bitternut hickory (*Carya cordiformis* (Wangenh.) K.Koch) and Caucasian wingnut (*Pterocarya fraxinifolia* (Lam). Spach.) (a new hostplant record), in addition to common walnut (*Juglans regia* L.). Interestingly, *C. lucifluella* is thought to have performed a host plant shift after its introduction into Europe, whereas *C. juglandiella* apparently did not. *Coptodisca juglandiella* has three generations in Hungary. The autumn generations of both species produced many more mines than the spring generations. One hymenopteran parasitoid specimen was reared from *C. juglandiella*. Larvae, mines and adults of *C. juglandiella* and *C. juglandiella* can be easily distinguished, differences are presented and illustrated. The genitalia of *C. juglandiella* are described for the first time.

Introduction

The family Heliozelidae (superfamily Adeloidea) contains 125 described species, with the highest diversity in North America and Australia (Milla et al. 2017; van Nieukerken et al. 2018). The host plant spectrum of Heliozelidae is wide: it includes deciduous trees, shrubs, vines as well as herbs (van Nieukerken and Geertsema 2015; Milla et al. 2017). The larvae are leafminers which, when fully fed, prepare oval cases (shields) from the leaves, in which pupation occurs. In temperate regions, they overwinter in the prepupal stage (Nieukerken et al. 2012). Altogether 12 species of Heliozelidae were known to occur in Europe until now (van Nieukerken et al. 2018), three of them being aliens from North America: *Antispila oinophylla* van Nieukerken & Wagner, 2012 (van Nieukerken et al. 2012), *Coptodisca lucifluella* (Clemens, 1860) (Bernardo et al. 2011b) and *C. splendoriferella* (Clemens, 1860) (Weiss 1918; Segerer et al. 2019). The first verified record of the 13th species, *C. juglandiella* (Chambers, 1874), another alien from North America, is reported in this paper.

The heliozelid genus *Coptodisca* Walsingham, 1895 is a New World taxon which includes 18 described species of tiny leafminer moths which feed on a wide variety of host plants (Bernardo et al. 2015). In 2010, a *Coptodisca* was found in Campania and Lazio (Italy) (Bernardo et al. 2011a, 2011b). Mines were found on common walnut (*Juglans regia* L.) and on black walnut (*J. nigra* (L.)). Specimens from *J. regia* were DNA barcoded and their identity, corroborated by morphological studies, was shown to be *Coptodisca lucifluella* (Bernardo et al. 2015). In its native range in North America, *C. lucifluella* feeds on hickory species (*Carya* spp.), but is only known from walnut (*Juglans* spp.) in the introduced range in Italy (Bernardo et al. 2015). The biology of *C. lucifluella* was extensively documented by Bernardo et al. (2015). Specimens from *J. nigra* were not DNA barcoded. *Coptodisca lucifluella* was subsequently also recorded from Hungary in 2017 (Takács et al. 2017), and in 2019 it was found in Ukraine, on *J. regia*, close to the Hungarian border (Pályi et al. 2019). Different host plant preferences and infection rates were detected in Hungary: all examined *J. nigra* trees were infected while we did not find mines on several *J. regia* trees. Where *J. nigra* and *J. regia* co-occurred, the former species was more frequently attacked than the latter by *Coptodisca* larvae at a ratio of 15:1 (Takács et al. 2017). We therefore suspected that two *Coptodisca* species could be present. Here, we show that our suspicion was correct and that two *Coptodisca* species are present in Hungary: *C. lucifluella*, a leafminer on *J. regia*, and *C. juglandiella*, with most occurrences on *J. nigra*. This paper presents the first records of *C. juglandiella* in Europe.

Material and methods

Field work, records and rearing

Since the discovery of *C. lucifluella* in 2017 in Hungary, we have searched for leaf mines on *Juglans regia* and *J. nigra* trees in 263 localities throughout the country, as well as on one tree each of white walnut (*J. cinerea* (L.)) and Arizona walnut (*J. major* (Torr.)). Additional specimens of the latter two species were not located. A locality is either a municipality (usually a village) or a district of Budapest. Food plants were examined until the first sighting of each *Coptodisca* species in each locality.

Coordinates of localities were compiled into the ETRS89 reference grid of 10×10 km squares. Mines with larvae were collected in August 2018 from *J. nigra* trees in the villages of Alcsútdoboz, Magyaralmás and Előszállás (all in Fejér county), *J. regia* trees in Cece and Székesfehérvár (Fejér county), from *J. major* in Alsószentiván (Fejér county, in 2019), from *J. cinerea* in Martonvásár (Fejér county), from bitternut hickory (*Carya cordiformis* (Wagenh.) K.Koch) in Budapest (2019), and from Caucasian wingnut (*Pterocarya fraxinifolia* (Lam.) Spach) also in Budapest, (2019). Mines found in 2018 were reared under natural conditions, each in a separate plastic vial, and hibernated outdoors. Mines collected in summer 2019 were kept at room temperature, with adults emerging within a week.

Adult material examined

Coptodisca juglandiella: 2 ♀: Fejér county, Alcsútdoboz, reared from *Juglans nigra*, collected: 15.viii.2018, emerged: 24.viii.2018, leg. Attila Takács, slide Nos TB1901f, TB1905f; 1 ♂: Fejér county, Alcsútdoboz, collected: 10.vi.2019, emerged: 19.vi.2019, leg. Attila Takács, slide No. TB1904m; 1 specimen [abdomen and head lost]: Fejér county, Alcsútdoboz, collected: 10.vi.2019, emerged: 19.vi.2019, leg. Attila Takács; 1 ♀, 1 ♂: Fejér county, Alsószentiván, *Juglans major*, collected: 28.vi.2019, emerged: 15.vii.2019, leg. Attila Takács, slide No. GP 29822 IgR, GP 29820 IgR; 1 ♂: Fejér

county, Alsószentiván, *Juglans nigra*, collected: 01.vi.2018, emerged: 23.vi.2018, leg. Attila Takács, slide No. GP 29763 IgR; 1 ♀: Fejér county, Magyaralmás, *Juglans nigra*, collected: 13.viii.2018, emerged: 14.viii.2018, leg. Attila Takács, slide No. TB1903f; 1 ♂: Somogy county, Öreglak, reared from *Juglans nigra*, collected: 19.viii.2018. emerged 27.viii.2018. (coll. Csaba Szabóky)

Coptodisca lucifluella: 1 ♀: Baranya county, Pécs, reared from *Juglans regia*, collected: 22.v.2018, emerged: 2.vi.2018, leg. Attila Takács, slide No. GP 29762 IgR; 3 ♂, 1 ♀: Zala county, Zalaszentgrót, reared from *Juglans regia*, collected: 31.vii.2018, emerged: 10.viii.2018, leg. Attila Takács, slide Nos TB1902m, TB1906m, TB1907m, TB1908f.

Adults were either pinned with minuten pins (n = 8) or glued to card points (n = 2) or stored unmounted in a small transparent capsule (n = 4). All adults are deposited in the Hungarian Natural History Museum (HNHM), unless stated otherwise.

Photography and genitalia preparation

Preimaginal stages were photographed by Miklós Bozsó and Attila Takács with a Leica MZ 7.5 stereomicroscope (Leica Microsystems); images were adjusted by Leica Application Suite (Leica Microsystems). Wings were kept in 10% KOH solution for two hours at room temperature. Isolated abdomens were boiled in 10% KOH solution for three minutes. All structures were mounted in euparal to prepare permanent microscope slides. Wings were stained with eosin. Wing preparations were photographed with an Olympus DP70 photographic microscope. Images were adjusted with the software Adobe Photoshop CS6.

Molecular analysis

DNA extraction

A total of ten specimens were selected for molecular analyses (Table 1). Specimens were killed and stored in 70% alcohol. The genomic DNA was isolated from two legs of adult specimens or from a piece of the larval body. DNA was isolated with Quick-DNATM Tissue/Insect Miniprep Kit (Zymo Research) according to the recommended protocol of the manufacturer.

PCR amplification and sequencing

Amplification of the 658 bp COI barcode region was performed with the primers LCO-1490 and HCO-2198 (Folmer et al. 1994). The PCR mixture contained 2 µl of template DNA, 7.5 µl of 2× HotStart Taq Plus Master Mix (HotStart Taq Plus Master Mix Kit, Qiagen), 0.5 µl of each primer (10 µM) and double distilled water to a final volume of 15 µl. The amplification profile consisted of a preheating step of 5 min at 95 °C followed by 5 cycles of 95 °C for 40 s, 45 °C for 1 min and 72 °C for 1 min, and additional 40 cycles at 95 °C for 40 s, 51 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 10 min. Reaction was performed in a PTC-100 DNA thermal cycler (MJ Research). Products of amplification were analysed in 2% agarose gel with addition of ethidium bromide and visualised under UV light. The PCR products were purified using the USB ExoSAP-IT PCR Product Clean-Up reagent (Affymetrix). Amplicons were sequenced bidirectionally using the BigDye Terminator Cycle Sequencing Kit (v3.1) (Applied Biosystems) on a MegaBase™ 1000 Sequencing System (GE Healthcare). Specimens used in this study are listed in Table 1 along with GenBank accession numbers (MN103407–MN103411). Sequences were inspected and translated in translate tool of ExPASy Bioinformatics Resource Portal (Artimo et al. 2012) to verify that they were free of stop codons.

Table 1. *Coptodisca* specimens from Hungary used for molecular analyses.

Species	Host plant	Locality	Longitude / Latitude	Date of collection	Date of emergence	Collector	NCBI GenBank code/BOLD System project code	Stage
C. lucifluella	*J. regia*	Zalaszentgrót	46°56.55'N, 17°04.9'E	31.vii.2018	10.viii.2018	János Kutas	MN103407/ JUG001-19	Adult
C. juglandiella	*J. nigra*	Alcsútdoboz	47°25.27'N, 18°35.28'E	12.viii.2018	24.viii.2018	Attila Takács	MN103410/ JUG004-19	Adult
	J. nigra	Alcsútdoboz	47°25.27'N, 18°35.28'E	12.viii.2018	24.viii.2018	Attila Takács	MN103410/ JUG004-19	Adult
C. lucifluella	*J. regia*	Székesfehérvár	47°12.47'N, 18°25.32'E	12.viii.2018	22.viii.2018	Attila Takács	MN103408/ JUG002-19	Adult
C. juglandiella	*J. nigra*	Magyaralmás	47°17.68'N, 18°19.78'E	27.vii.2018	14.viii.2018	Attila Takács	MN103410/ JUG004-19	Adult
	J. nigra	Előszállás	46°49.72'N, 18°49.53'E	22.vii.2018	04.viii.2018	Attila Takács	MN103411/ JUG005-19	Adult
C. lucifluella	*J. regia*	Cece	46°45.98'N, 18°38.32'E	12.viii.2018	04.viii.2018	Attila Takács	MN103409/ JUG003-19	Adult
C. juglandiella	*J. nigra*	Előszállás	46°49.72'N, 18°49.53'E	12.viii.2018	04.viii.2018	Attila Takács	MN103410/ JUG004-19	Adult
	J. major	Alsószentiván	46°47.75'N, 18°43.97'E	05.vii.2019	n/a	Attila Takács	MN103410/ JUG004-19	Larva
	J. cinerea	Martonvásár	47°19.03'N, 18°46.75'E	23.ix.2019	n/a	Mátyás Papp	MN103410/ JUG004-19	Larva

Sequence analysis and phylogenetic reconstruction

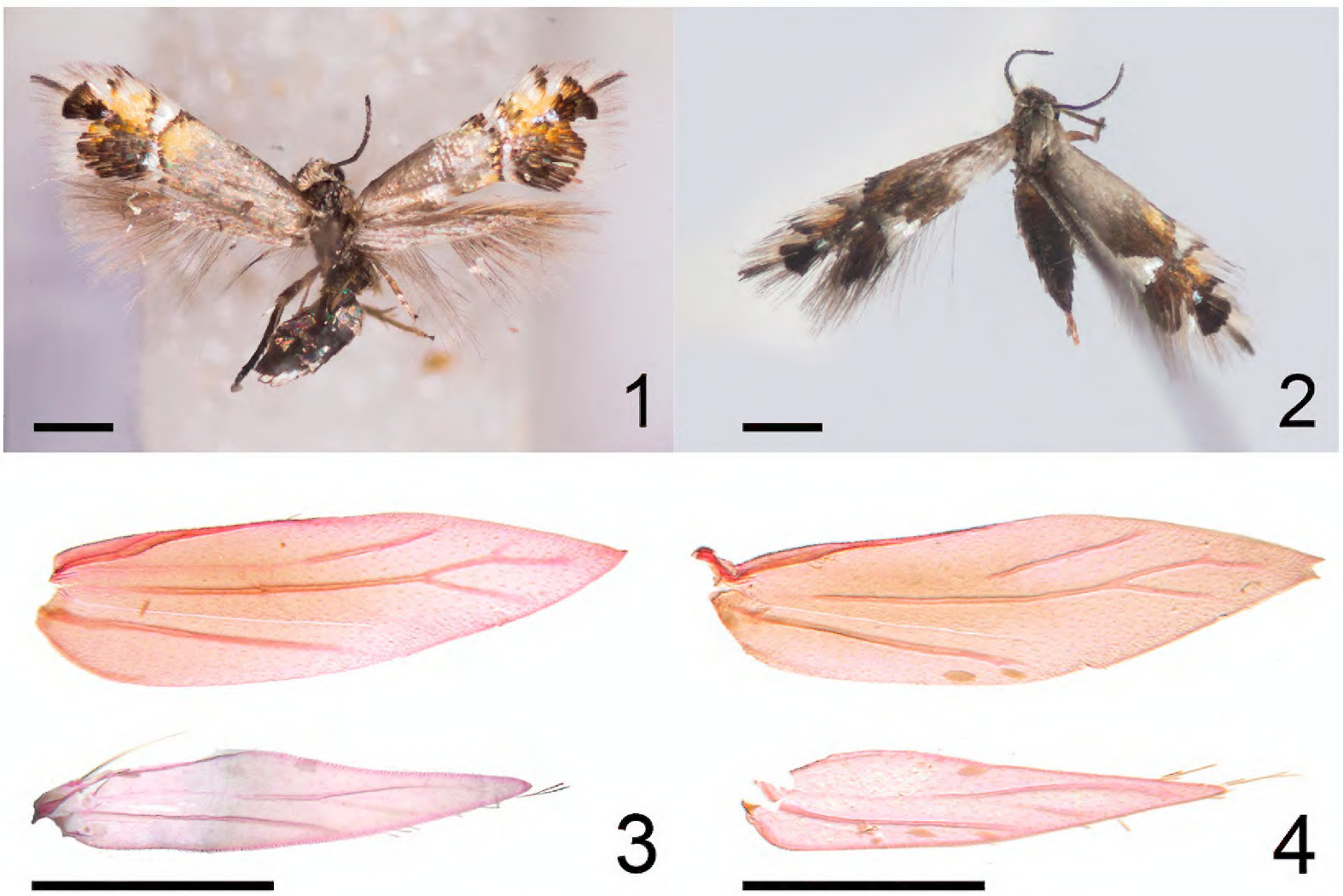
The forward and reverse sequences were assembled with Staden Package 2.0.0b9. Our purpose was to use as many *C. lucifluella*, *C. juglandiella* sequences suitable for aligning as possible for our analysis (we found eight additional available unique sequences for these species from NCBI GenBank and BOLD Systems) (Bernardo et al. 2015; van Nieukerken et al. 2012). The multiple sequence alignment was carried out by ClustalW (Larkin et al. 2007), with default parameters for all available corresponding gene sequences of *C. lucifluella* and *C. juglandiella* and with outgroup taxa (*Antispila argentifera* Braun, 1927). Distance analysis within examined taxa was obtained based on uncorrected p-distances using MEGA 7 (Kumar et al. 2016).

The most appropriate model of nucleotide substitution was determined with MEGA under the Bayesian Information Criterion (BIC). The Tamura 3 parameter (T92) model with gamma distribution (G) (Tamura and Nei 1993) was selected for our phylogenetic reconstruction. Phylogenetic trees were constructed with the Maximum Likelihood (ML) method implemented in MEGA, starting from a random Neighbor Joining tree, with the default initial rearrangement settings and five discrete gamma categories. To obtain an estimate of the support for each node, a bootstrap analysis using 1000 replicates was performed. Bootstrap support is given on appropriate clades in the ML tree.

Results

Coptodisca juglandiella

Identification. The original description of *C. juglandiella* contains little information about the habitus of the moth ("Except that it is *perhaps* a trifle smaller, I cannot distinguish this species in



Figures 1–4. External morphology of *Coptodisca* species from Hungary. **1**. Habitus of *C. juglandiella* male: Somogy county, Öreglak, reared from *Juglans nigra* (coll. Csaba Szabóky), **2**. *C. lucifluella* female: Zala county, Zalaszentgrót, reared from *Juglans regia* (coll. HNHM). **3**. Denuded right wings of *C. juglandiella* (TB1903f). **4**. Denuded right wings of *C. lucifluella* (TB1902m). **1, 2**: photo by Attila Takács, **3, 4**: photo by Balázs Tóth. Scale bars: 0.5 mm.

the imago from *A. splendoriferella*."); it deals rather with the mine, the case and host plant (Chambers 1874). On the contrary, Clemens (1860) gives a detailed description of the adult of *C. lucifluella*, but this is still not sufficient for identification. Bernardo et al. (2015) redescribed this species, designated and figured its lectotype and figured also an adult specimen of *C. juglandiella* and gave a brief diagnosis of the latter species. We did not have the opportunity to examine the type specimen(s) of *C. juglandiella*. According to Miller & Hodges (1990) the majority of Chambers' types are deposited in the Museum of Comparative Zoology (Harvard University) but a significant number are located at the National Museum of Natural History (Smithsonian Institution, Washington DC = USNM) and there are also potential but probably untraceable type specimens at The Natural History Museum (London). This species is absent from the list of Miller and Hodges (1990). We consulted the type database of USNM without result (NMNH 2020), thus the type(s) may be lost. Hungarian specimens of the two species show the diagnostic differences underlined by Bernardo et al. (2015). There is an image of an intact *C. juglandiella* specimen on the homepage of the Moth Photographers Group (2019) which matches the diagnosis and image of Bernardo et al. (2015, suppl. material 3). Therefore, we are confident that the identifications of Hungarian specimens as *C. lucifluella* and *C. juglandiella* are correct.

Diagnosis. Adults of *C. juglandiella* (Fig. 1) are similar in external morphology to *C. lucifluella* (Fig. 2). However, the two species can be distinguished by wing pattern: the conspicuous black

filling of *C. lucifluella* between the silvery grey basal area and the patch at dorsum is absent in *C. juglandiella*, in which the colour of this area is transitional from silvery grey (proximal edge) to yellow (distal edge). The silvery white tornal patch is narrower in *C. juglandiella* than in *C. lucifluella*. No differences in wing shape and venation were found between the two species (Figs 3, 4). In the male genitalia, we found more sensilla of the pectinifer in *C. juglandiella* (Fig. 5a, b) than in *C. lucifluella* (Fig. 6a, b), while differences between the female genitalia of the two species were not observed.

The shape of the mines is different in the two species: in *C. juglandiella* mines are always round, 7 mm long and 5 mm wide on average (Fig. 11), mines of *C. lucifluella* are rather elongate and angular (Figs 9, 10), average size 8 mm × 5 mm. The position of mines in leaflets is also different: mines of *C. juglandiella* are situated mostly close to the junction of the midrib and a lateral vein (Fig. 13), while the mines of *C. lucifluella* may occur throughout the leaflet (Fig. 12). Frass of the larva of *C. juglandiella* is hidden under a small area of the upperside parenchyma of the leaflet, preserved by the larva (Fig. 11), in contrast to the frass of *C. lucifluella* which is visible from both sides because it is dispersed in the mine and no cover is preserved (Fig. 10).

The body of the mature larva of *C. juglandiella* is off-white and its head, as well as the first two thoracic segments, are pale yellow (Fig. 15) while the larva of *C. lucifluella* is monochromatic rusty brown (Fig. 14).

We could not observe differences in the morphology of the cases or the pupae between the two species.

Morphology and recorded host plants. ***Adult.*** External characters (Figs 1, 3). Length of forewing 1.5–1.8 mm including fringe. Head silvery grey, rest of body dark silvery grey. Basal half of forewing silvery grey; two triangular patches present at distal thirds of costa and dorsum, these patches silvery white with black border at each side. Colour of the area between the silvery grey basal field and the patch at dorsum transitional from silvery grey to yellow in proximo-distal direction. Patch of dorsum narrow (Fig. 1).

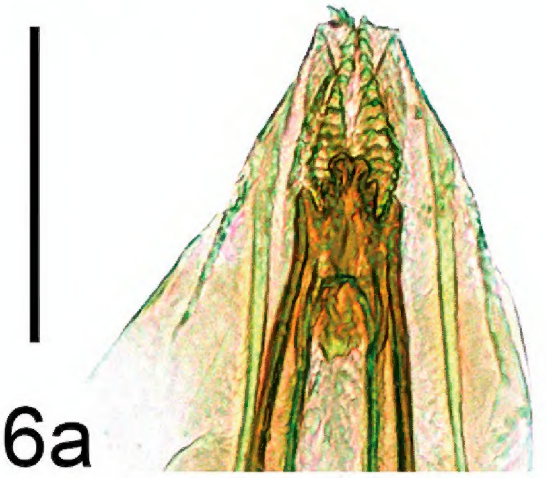
Genitalia. In the male genitalia the pectinifer has six sensilla on right valva and seven sensilla on left valva (Fig. 5a, b). Phallus with two rows of anellar spines. Female genitalia with six oviscapt teeth: a pair of large ones anteriorly, posterior ones forming a broad, slightly indented apex (Fig. 6a). Corpus bursae ovoid, apparently without either scobinations or signum (Fig. 6b).

Mine. *Coptodisca juglandiella* prepares blotch mines (Fig. 11) visible on both sides of the leaf. Mines are always situated between lateral veins and never traverse them. The shape of mines is always round, 7 mm long and 5 mm wide on average. Mines usually start close to the junction of the midrib and a lateral vein. This species preserves a small area of the upperside palisade parenchyma of the leaflet and hides all of its frass under this cover.

Larva. Body off-white, head and first two thoracic segments pale yellow (Fig. 15).

Case. Prepared from an oval-shaped area of the leaflet, which is severed from the leaf by chewing the epidermis on both sides around this piece (Fig. 19). Then the oval plates are attached with silk along the edges, forming an oval-shaped shield, which is somewhat more elongate than the original leaf pieces, 2.9 mm long and 1.3 mm wide (Fig. 18).

Host plants. *Coptodisca juglandiella* is usually found on *Juglans nigra* in Hungary, but we also found it on *J. major* and *J. cinerea* (new hostplant records).



Figures 5, 6. Genitalia of *Coptodisca juglandiella* from Hungary, Alsószentiván, reared from *Juglans nigra*. **5a**. Enlarged region of uncus, tegumen and valvae of male genitalia, lateral view (GP 29763 IgR). **5b**. Ventral view of male genitalia (GP 29763 IgR). **6a**. Enlarged region of oviscapt of female genitalia (GP 29822 IgR). **6b**. Female genitalia (data as **6a**). Photos by Ignác Richter. Scale bars: 0.1 mm (**5a, 5b, 6b**), 0.05 mm (**6a**).

Coptodisca lucifluella

Morphology and recorded host plants. ***Adult.*** External characters (Figs 2, 4). Length of forewing 1.5–1.8 mm including fringe. Head silvery grey, rest of body dark silvery grey. Basal half of forewing silvery grey, with two triangular patches present at distal thirds of costa and dorsum, white with black border on each side. Area between silvery grey basal field and tornal patch dark fuscous. Tornal patch broad, especially at base (Fig. 2).

Genitalia (Figs 7, 8). Male genitalia with five sensilla on pectinifers of both valvae (n = 2; right valva of an additional specimen lost, left valva with five teeth), elsewhere 5–7 sensilla (Bernardo et al. 2015, see fig. 7). Phallus with two rows of spines. Female genitalia with six oviscapt teeth, a pair of large ones anteriorly, posterior ones forming a wide, slightly indented apex (Fig. 8a). Corpus bursae rather pyriform, with neither scobinations nor signum (Fig. 8b).

Mine. This species prepares blotch mines visible on both sides of the leaf (Figs 9, 10). Mines are always situated between lateral veins and never traverse them. The shape of mines is rather elongate and angular (Fig. 10), average size 8 mm × 5 mm. Mines are positioned close to lateral veins, equally distributed from the midrib to the edge of the leaflet (Fig. 12). The frass of *C. lucifluella* is visible from both sides as it is dispersed in the mine (Fig. 9).

Larva. Mature larva 2.5 mm long, monochromatic rusty brown in colour (Fig. 14).

Case. Prepared from an oval-shaped area of the leaflet, severed from the leaf by chewing the epidermis on both sides around this piece (Fig. 19). Then the oval plates are attached with silk along the edges, forming an oval-shaped shield, which is somewhat more elongate than the original leaf pieces, 2.9 mm long and 1.3 mm wide (Fig. 18).

Pupa. Fresh pupa pale yellow, then becoming dark yellow, 2.7 mm long (Fig. 17).

Host plants. Host plants of *C. lucifluella* in Hungary are *Juglans regia*, *Carya cordiformis* and *Pterocarya fraxinifolia* (new hostplant record).

Remarks. Genitalia images prepared by us have a resolution too low for publication, but the above-mentioned characters are still observable. We illustrate here the genitalia with images kindly sent by Erik J. van Nieukerken (Fig. 7a, b; partly from Bernardo et al. 2015).

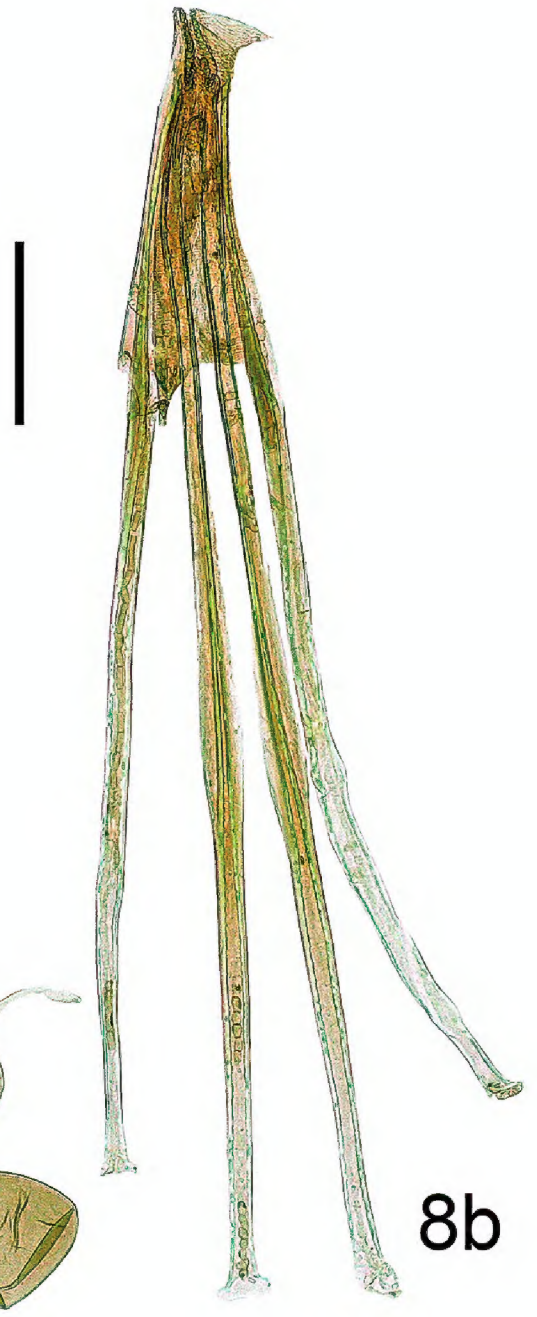
Records of *Coptodisca* species in Hungary

At least one *Coptodisca* species was present altogether in 227 localities, see Figs 20, 21. *Coptodisca juglandiella* was found in 31 localities while *C. lucifluella* was present in 216 localities. See Suppl. material 1 for the complete list of municipalities with data of sightings, including coordinates for the individual trees.

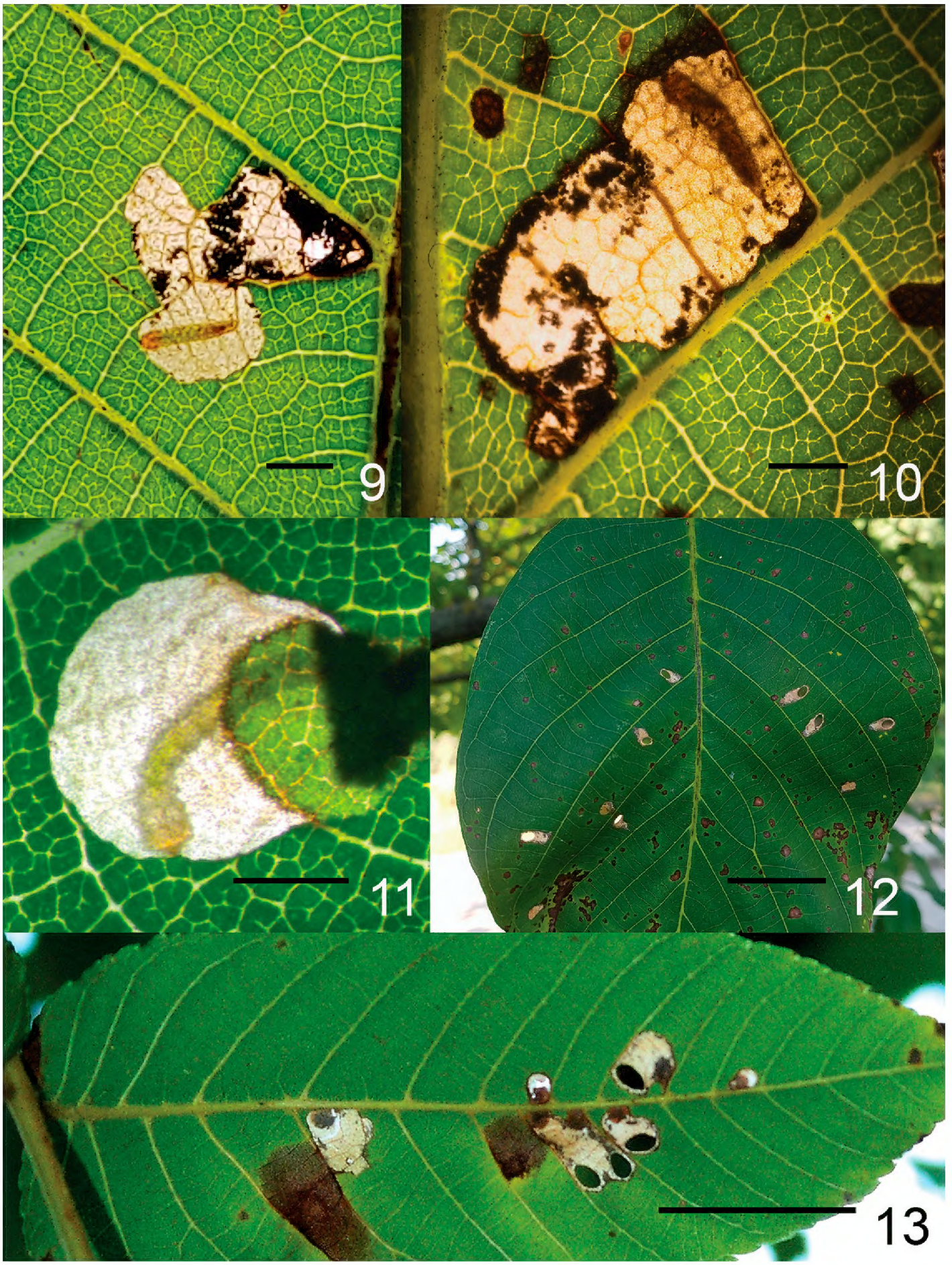
Life cycle of *Coptodisca* species in Hungary

Adults of the first generation emerge from mid-May to late May in both species. We found one mine per leaflet in the first generation, while there were three to eight mines present on each leaflet in the last generation, in both species.

Larvae consume the mesophyll of the leaf (Figs 9–11). The development time of larvae was eight to 18 days. Fully grown larvae made their case from the epidermis of the upper- and undersides of the leaflet (Fig. 19), then descended to the lower leaves of host plants by silken threads in the first half of June. They attached their case to those lower leaves or to the fruit. Pupation occurred in the case, and the pupal stage lasted for five to eight days. From June to October we



Figures 7, 8. Genitalia of *Coptodisca lucifluella*. **7a**. Enlarged view of pectinifers of male genitalia, with five sensilla on left valva and six sensilla on right valva. Ventral view (from Bernardo et al. 2015, Italy, reared from *J. regia*, slide No. EvN 4462, coll. RMNH) **7b**. Male genitalia with six sensilla on left valva and seven sensilla on right valva, semi-ventral view, phallus at right in ventral view (photos of male genitalia provided by Erik van Nieukerken, photograph of phallus from Bernardo et al. 2015, U.S.A., moth reared from *Carya tomentosa*, slide No. EvN 4458, coll. USNM). **8a**. Enlarged region of oviscapt of female genitalia (Hungary, Pécs, 02.vi.2018, slide No. GP 29762 IgR). **8b**. Female genitalia (data as **8a**). **7a, 7b**: photo by Erik van Nieukerken, **8a, 8b**: photo by Ignác Richter. Scale bars: 0.1 mm (**7a, 7b, 8b**), 0.05 mm (**8a**).



Figures 9–13. Blotch mines of *Coptodisca* species in Hungary. **9**. Mine of. *C. lucifluella* with larva, frass dispersed. **10**. Another mine of. *C. lucifluella* with larva, higher magnification than **9**. Both mines on *J. regia*, Tornyiszentmiklós, 27.viii.2017. **11**. Mine of *C. juglandiella* with larva, frass covered by a small area of the upper parenchyma. On *J. nigra*, Fejér county, Alcsútdoboz, 21.v.2018. **12**. Vacated mines of *C. lucifluella*, scattered throughout the leaflet. On *J. regia*, Fejér county, Lovasberény, 14.x.2019. **13**. Vacated mines of *C. juglandiella*, close to midrib. On *J. nigra*, Fejér county, Alcsútdoboz, 19.viii.2019. Photos by Miklós Bozsó. Scale bars: 2 mm (**9–11**), 20 mm (**12, 13**).

Figures 14, 15. Mature larvae of *Coptodisca* species from Hungary. **14**. *C. lucifluella*, on *J. regia*, Fejér county, Lovasberény, 15.x.2019. **15**. *C. juglandiella*, on *J. nigra*, Fejér county, Alcsútdoboz, 29.viii.2018. Photos by Attila Takács. Scale bars: 0.5 mm.

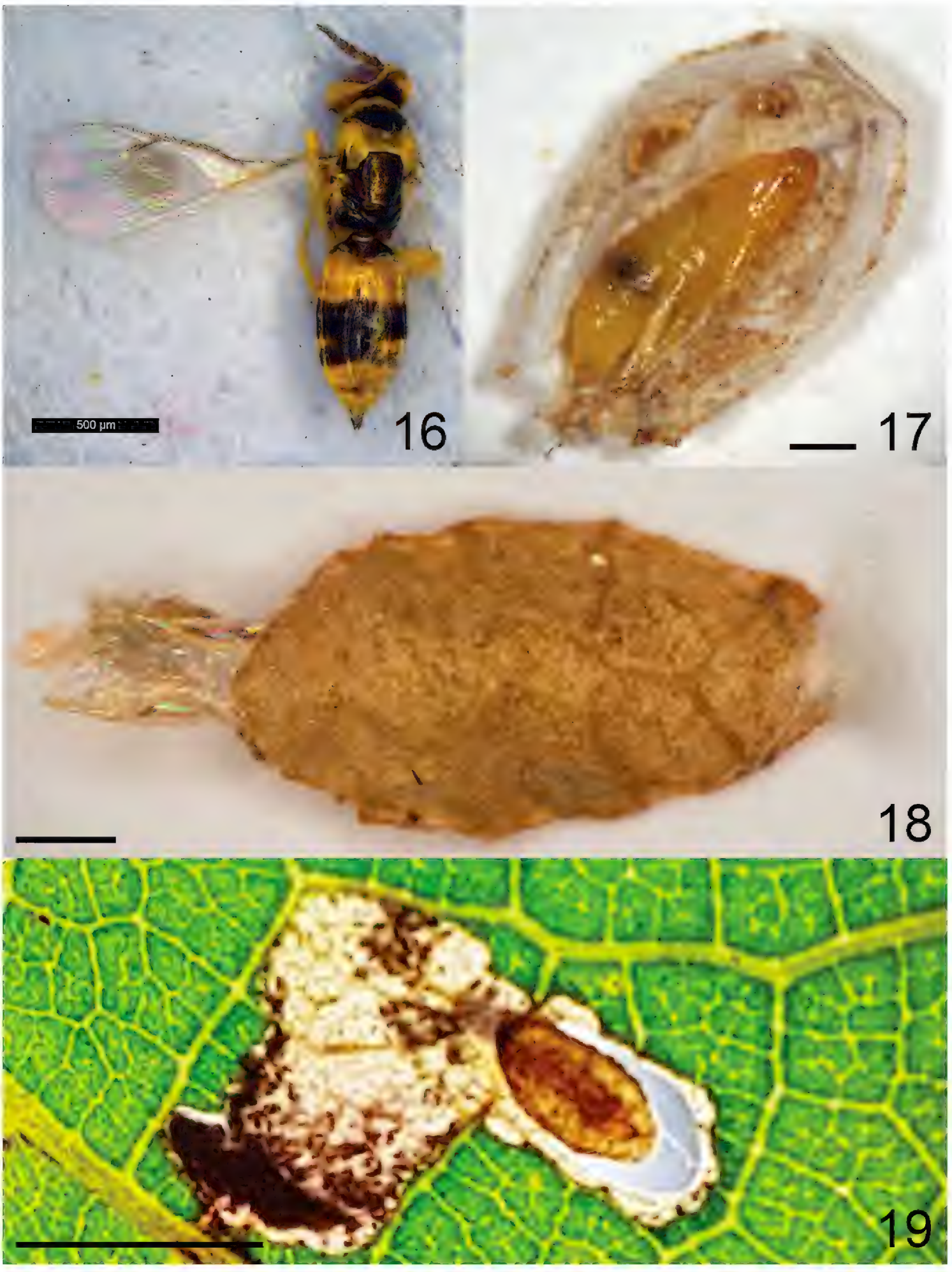
continuously found active mines (living larvae). Larvae preparing for hibernation descended to twigs or the trunk of the host plant – or even to other nearby plants – by silken threads in September and October and attached their cases to those surfaces where overwintering occurred. Mortality of larvae was very high in this stage, and most larvae died in their mines. The causes of mortality were not studied. Successfully overwintered larvae pupated in late April or early May. Altogether 23 adults of *C. lucifluella* and 13 adults of *C. juglandiella* emerged.

Molecular analysis

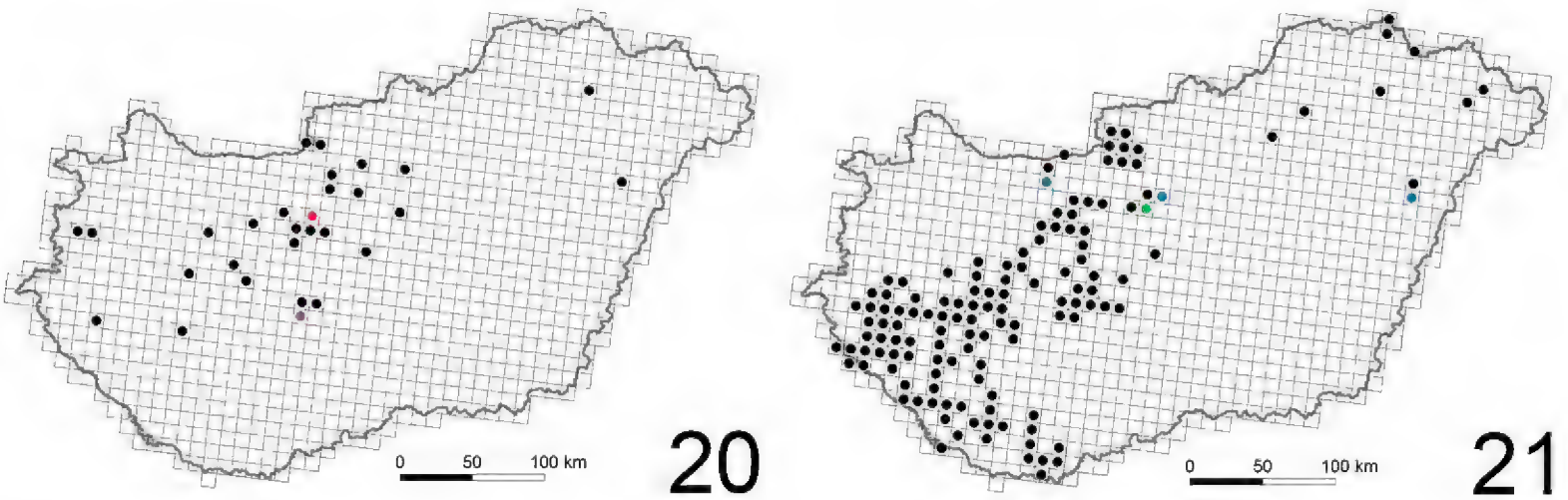
The alignment of the COI counted 425 positions in the final dataset. Relationships among examined taxa and populations inferred from the analysed mtDNA region are shown in Fig. 22.

Based on results of our analysis, the intraspecific mean distance of the common clade of "*lucifluella*" populations was 0.9% (less than 4 basepairs). The "*lucifluella*" clade divided into two subclasses according to our data. Two Hungarian (Zalaszentgrót, Székesfehérvár) populations formed a common clade with other studied American, Canadian and Italian populations. The third Hungarian population (Cece) and one of the studied American population (KJ427015) probably formed a common clade with the same sequence based on our analysis.

In the case of *C. juglandiella* the examined seven specimens from five populations were characterised by two sequences. This was explained by the fact that six specimens – two specimens from Alcsútdoboz, one specimen from Magyaralmás, Előszállás, Alsószentiván and Martonvásár



Figures 16–19. Parasitoid wasp, cases and pupae of *Coptodisca* species from Hungary. **16**. Unidentified *Closterocerus* Westwood, 1833 species, female, reared from *C. juglandiella*. Alcsútdoboz, 22.vi.2018. **17**. Pupa of *C. lucifluella*, in an opened case from *J. regia*, Zala county, Tornyiszentmiklós, 27.viii.2017. Photo by Miklós Bozsó. **18**. Case with exuviae, *C. juglandiella*, from *J. nigra*, Fejér county, Alcsútdoboz, 29.viii.2019. **19**. Case just finished, *C. lucifluella*, on *J. regia*, Zala county, Tornyiszentmiklós, 27.viii.2017. Photos by Attila Takács. Scale bars: 0.5 mm (**16–18**), 5 mm (**19**).



Figures 20, 21. Collecting sites of *Coptodisca* species in Hungary mapped on 10×10 km squares. **20**. *C. juglandiella*: black dot = mines on *Juglans nigra*, red dot = mines on *J. cinerea*, purple dot = mines on *J. major*. **21**. *C. lucifluella*: black dot = mines on *J. regia*, green dot = mines on *Carya cordiformis*, blue dot = mines on *Pterocarya fraxinifolia*.

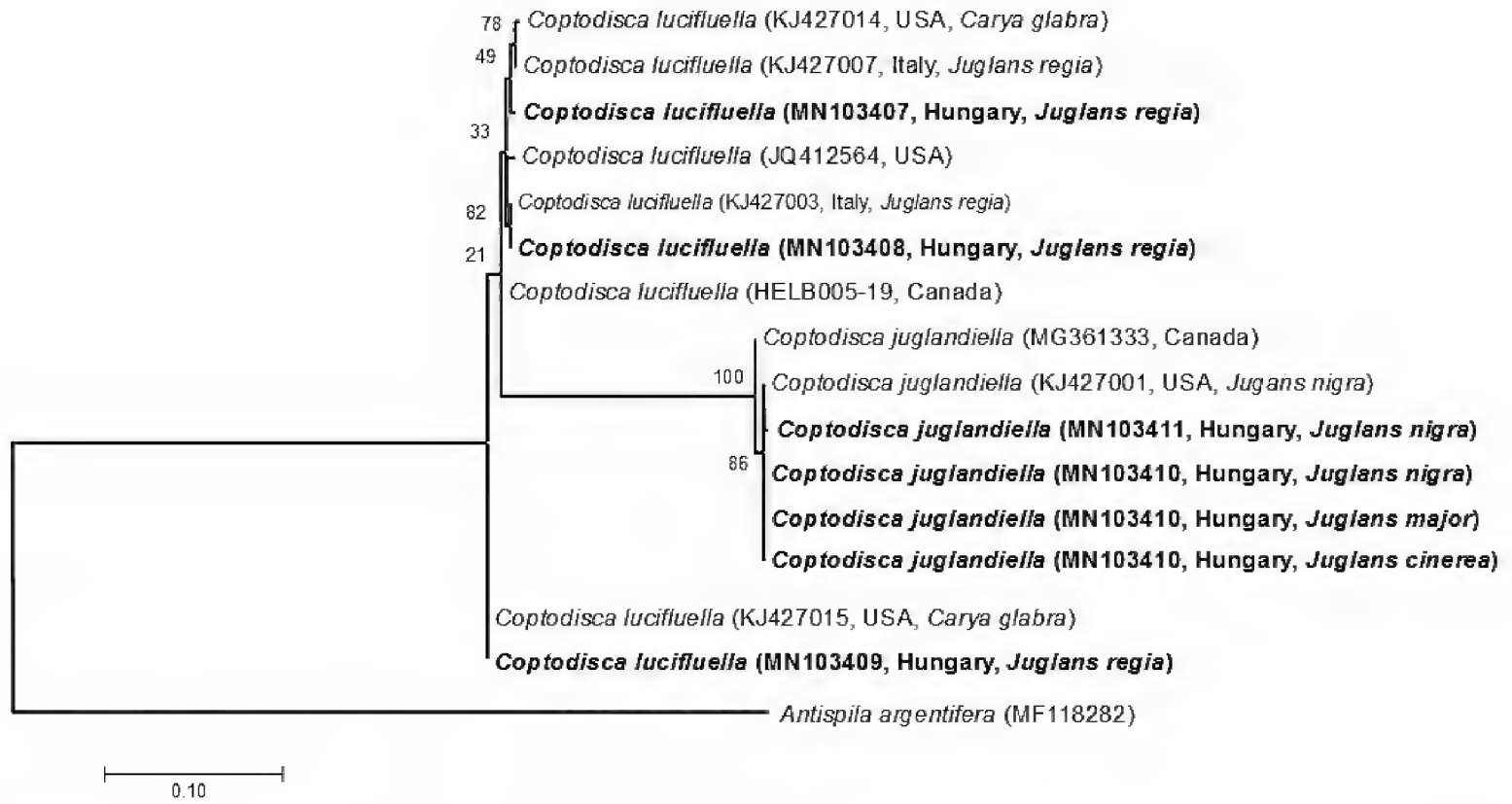

78
Coptodisca lucifluella (KJ427014, USA, Carya glabra)
49
Coptodisca lucifluella (KJ427007, Italy, Juglans regia)
Coptodisca lucifluella (MN103407, Hungary, Juglans regia)
33
Coptodisca lucifluella (JQ412564, USA)
82
Coptodisca lucifluella (KJ427003, Italy, Juglans regia)
21
Coptodisca lucifluella (MN103408, Hungary, Juglans regia)
Coptodisca lucifluella (HELB005-19, Canada)
Coptodisca juglandiella (MG361333, Canada)
100
Coptodisca juglandiella (KJ427001, USA, Jugans nigra)
Coptodisca juglandiella (MN103411, Hungary, Juglans nigra)
86
Coptodisca juglandiella (MN103410, Hungary, Juglans nigra)
Coptodisca juglandiella (MN103410, Hungary, Juglans major)
Coptodisca juglandiella (MN103410, Hungary, Juglans cinerea)
Coptodisca lucifluella (KJ427015, USA, Carya glabra)
Coptodisca lucifluella (MN103409, Hungary, Juglans regia)
Antispila argentifera (MF118282)
0.10

Figure 22. Phylogenetic relationships of examined *C. juglandiella* and *C. lucifluella* populations. The best likelihood phylogenetic tree was inferred from the COI sequence (425 bp) alignment under the T92+G model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Species name, GenBank or BOLD Accession number are listed for each taxon. Sequences of Hungarian *Coptodisca* populations are indicated in bold. The scale bars represent the number of substitutions per site.

– had the same sequence (MN103410). The intraspecific mean distance of the common clade of "*juglandiella*" populations was 0.3% (less than 2 basepairs). The interspecific mean distance between studied *Coptodisca* taxa was 8.5% (36 basepairs).

Parasitoids

Altogether 40% of cases we found during field work had a round exit hole of a parasitoid insect. In rearing, only one female of an unidentified *Closterocerus* Westwood, 1833 (Hymenoptera: Eulophidae) (Fig. 16) emerged from a *C. juglandiella* case. Data of the specimen: Fejér county, Alcsútdoboz, emerged: 22.vi.2018., leg. Attila Takács (to be deposited in HNHM). We did not find additional parasitized larvae.

Discussion

Each newly introduced species should be examined with special regard to its tendency of invasiveness in order to explore any possible negative effects on indigenous ecosystems. We lack knowledge of the life cycles and natural enemies of the majority of alien species (Sundseth et al. 2019), and this is also true for *C. juglandiella*. Colonisation by an invasive species can be devastating and it can seriously reduce the populations of its host plant. In Hungary, the leaves of horse chestnut (*Aesculus hippocastanum* (L.)) trees fall already in mid-August due to combined effects of the mines of *Cameraria ohridella* Deschka et Dimić, 1986 and the induced fungal diseases (Tuba et al. 2012, pp. 96–97). This damage forces the tree to sprout, utilising its winter buds (which are also flower buds), hence degrading its condition. The resulting stress can lead to death of the tree.

In the genitalia of *Coptodisca* species we found some differences compared to the results of Bernardo et al. (2015). They found that in the male genitalia of *C. lucifluella* the pectinifer of the left valva contains one more sensillum than that of the right valva, but our two examined specimens have five sensilla in the pectinifer of each valva. Lafontaine (1974) included the number of pectinifer teeth in his description of *C. matheri* Lafontaine, 1974 but Opler (1971), in the diagnosis of *C. powellella* Opler, 1971 omitted it, and referred to the aedeagus (= phallus) bearing "the most obvious differences". He illustrated the genitalia of his new species together with those of *C. quercicolella* Braun, 1927; according to these line-drawings the number and shape of sensilla are different in these two species. In the female genitalia of our *C. lucifluella* specimen the number of oviscapt teeth are the same as in the specimens of Bernardo et al (2015). The differences we observed in the genitalia of *C. lucifluella* versus *C. juglandiella* are to be interpreted with reservation due to (1) the very low sample size and (2) the fact that a taxonomic revision on this group is still lacking.

The examination of DNA barcodes confirmed that there are two *Coptodisca* species feeding on *Juglans* trees in Hungary. Our phylogenetic analysis showed that the populations of the studied *Coptodisca* taxa did not form well-separated clades in all cases, some separation was only weakly supported. The Hungarian *C. lucifluella* populations clustered in a common clade with populations from Italy, Canada and the USA. This clade seems to be separated from its closely related sister clade which contains all studied *C. juglandiella* populations. In both species, we observed that the sequences of some Hungarian populations were identical to sequences of Italian or American specimens. These observations may be supporting the scenario of two recent introductions from the Nearctic.

Although *C. lucifluella* in Italy was also recorded from mines on *J. nigra*, no specimen was barcoded from that host plant (Bernardo et al. 2015), so the identity of that population needs to be re-examined. *Coptodisca lucifluella* is found on *J. regia*, *C. cordiformis* and *P. fraxinifolia* in Hungary while *C. juglandiella* feeds on *J. major*, *J. nigra* and *J. cinerea*. *Juglans nigra* was previously known as host plant for *C. juglandiella* (e.g. Bernardo et al. 2015; Eiseman 2019) while *J. major* and *J. cinerea* are new host plants for this species.

Juglans species are not indigenous in Hungary. *Juglans regia* was introduced when Transdanubia was part of the Roman Empire as Pannonia. *Juglans nigra* appeared in botanical gardens in the 18th century (Bencat 1982), but became widespread in the last 50 years, cultivated for its wood. *Carya* and *Pterocarya* species occur only as ornamental trees in Hungary, mainly in botanical gardens. We are not able to trace back when the *Coptodisca* species appeared in Hungary: at the time of discovery they were already widespread.

The native host plant of *C. juglandiella* in North America is *J. nigra*. As it simply found the same plant species in Hungary, it was able to spread rapidly. For *C. lucifluella*, the situation is different: individuals of its native host plant in Europe are very isolated and thus the moth could have limited chance of expansion without a host plant shift. *Juglans regia*, the main European host plant of *C. lucifluella*, is not indigenous in North America. Host plant shift was suggested by Bernardo et al. (2011) to explain the presence of *C. lucifluella* in Italy, however, we still do not have enough data to determine the time and place of that event.

At least three generations of *C. juglandiella* can develop per year in Hungary but, due to the continuous presence of active mines from June to October, the generations appear to overlap and it is difficult to establish their real number. We have shown that *C. juglandiella* and *C. lucifluella* have been present in Hungary for several years and occur now all over the country. While these species were extending their range, they remained undetected until recently. Kremer et al. (2008) listed 17 European countries where *J. nigra* is present, and *C. juglandiella* should be searched for in these states.

The parasitoid specimen belongs to a eulophid (Hymenoptera) genus that is distributed in the Holarctic and Australian Regions, with several species specialised on leafminers of various insect orders (Protasov et al. 2007).

Damage by *Coptodisca* larvae is not a subject of concern yet, but a constellation of unfavourable weather conditions and a large population explosion could cause deterioration of the condition of *Juglans* trees due to leaf surface loss.

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Supplementary material 1

Table S1. Nearctic walnut leafminers invade Europe: First *Coptodisca lucifluella* (Clemens. 1860) and now *C. juglandiella* (Chambers. 1874) (Lepidoptera: Heliozelidae)

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Data type: Table.

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